In the Specification:

Please replace the paragraph beginning at page 6, line 25, with the following:

--**Figure 2.** Nucleotide coding sequence (SEQ ID NO:1) for the mature form of TEM-1 β-lactamase and the encoded amino acid sequence (SEQ ID NO:2) (Sutcliffe, *Proc Natl Acad Sci* (1978) *75*:3737). From the sequence for plasmid pBR322 (SYNPBR322), Genbank accession no. J01749. The break-points between the α and ω fragments at residues Asn52/Ser53, Glu63/Glu64, Gln99/Asn100, Pro174/Asn175, Glu197/Leu198, Lys215/Val216, Ala227/Gly228 and Gly253/Lys254 are indicated.--

Please replace the paragraph beginning at page 7, line 17, with the following:

--**Figure 6.** Vectors and strategy for the expression of heterologous proteins as fusions to the α197 and ω198 fragments of TEM-1 β-lactamase for interaction-dependent β-lactamase activation by fragment complementation. Vector pAO1 is a high-copy pUC119-based phagemid for expression of ω198 fusions and free ligands from dicistronic transcripts, which can be rescued as phage for quantitative introduction into host cells by high-multiplicity infection. Vector pAE1 is a low-copy p15A replicon with a strong promoter for expression of α197 fusions at comparable or higher levels than expression from the pAO1 vector. Trxpeps are 12-mer peptides inserted into the active site of thioredoxin. Tripep-trx libraries are random tri-peptides at the N-terminus of thioredoxin with an intervening Gly_4Ser (SEQ ID NO:3) linker. ScFv, single-chain antibody Fv fragment. LC-CH1, antibody fragment composed of light chain and first constant region of heavy chain. VL, antibody light chain variable region. *lac* prom, lactose operon promoter. SP, signal peptide. $(Gly_4Ser)_3$ (SEQ ID NO:4), flexible 15-mer linker. pUC ori, p15A ori, plasmid origins of replication. f1 ori, filamentous phage origin of replication. *cat*, chloramphenicol resistance gene. m.o.i., multiplicity of infection. *trc* prom, fusion promoter from tryptophan and lactose operons. *tt*, transcription terminator.

kan, kanamycin resistance gene. Vector sizes in base pairs (bp) do not include interactors.--

Please replace the paragraph beginning at page 8, line 15, with the following:

--Vectors and protocol for construction of a multiplex protein-protein interaction library using interaction-dependent β-lactamase fragment complementation systems. Expressed sequence (ES), i.e., random-primed cDNA libraries, are subcloned into phagemid vectors for expression as fusions to the β-lactamase α and ω fragments, via the flexible linker $(Gly_4Ser)_3$ (SEQ ID NO:4). The vectors encode a peptide epitope tag, such as the 12-residue Myc tag, at the C-terminus of the ES. When co-expressed with anti-Tag scFv, such as anti-myc 9E10, fused to the other fragment, the ES libraries can be selected for β-lactamase activity driven by the Tag-anti-Tag interaction, which will require stable expression of the ES fragment. The resultant libraries, enriched for stable expressors of autonomously folding domains (AFD), may then be rescued as phage and co-infected into male cells for selection of interacting AFD pairs (Multiplex Interaction Library). The AFD libraries can also be co-infected with scFv libraries, antibody light chain variable region libraries (VL), or peptide libraries displayed on thioredoxin (trx-peptide) for simultaneous selection of binding proteins for each AFD (Multiplex Antibody/Peptide Binder Selection). See legends to Figures 6 and 10 for identification of other abbreviations.--

Please replace the paragraph beginning at page 8, line 31, with the following:

--**Figure 10.** Abbreviated output of the PredictProtein Program for prediction of secondary structure and solvent exposure for NPTII (Rost and Sander, 1993, 1994). The top line shows the amino acid sequence in single letter code (SEQ ID NO:7). The second and third lines show secondary structure prediction. H, helix; E, strand; L, loop. The fourth line shows a measure of reliability on a scale from 1 to 10, with 10 being highest.

The fifth line shows solvent accessibility – e, exposed; b, buried. The bottom line shows a measure of reliability for solvent accessibility on a scale of 1 to 10, with 10 being highest. Ten regions of the sequence predicted to have little secondary structure and to be exposed to solvent are indicated by underlining as potential sites for productive fragmentation.--

Please replace the paragraph beginning at page 9, line 9, with the following:

--**Figure 11.** Expression vectors for production of β-lacα253 and β-lacω254 fusion proteins with scFv. Arrows denote translation start sites. T7 prom, bacteriophage T7 promoter; SP, pelB signal peptide; scFv is comprised of VH (antibody heavy chain variable region), $(Gly_4Ser)_3$ (SEQ ID NO:4) (15-mer flexible linker), and VL (antibody light chain variable region); *kan*, kanamycin resistance; His_6 (SEQ ID NO:5), hexa-histidine tag for metal ion affinity purification; $lacI^q$, high-affinity *lac* operon repressor mutant; f1 ori, phage origin of replication.--

Please replace the paragraph beginning at page 22, line 29, with the following:

--The first step in the development of high-performance enzyme fragment complementation systems is to construct vectors to express each fragment in the fragment pair library. A convenient system for selective fragment library expression may be derived from the expression system illustrated in Figure 6. All fragment pairs regardless of the intended application can potentially benefit from and would not be impaired by the docking function provided by interactors such as the fos and jun helixes fused to the break-point termini. Thus, the C-terminal, or ω fragment library would be expressed as N-terminal fusions via a flexible polypeptide linker such as a $(Gly_4Ser)_3$ (SEQ ID NO:4) linker to the fos helix (Interactor 2 in Figure 6) from the *lac* promoter in the phagemid vector pAO1 (the upstream cistron could be removed if desired). The amino acid sequence of the flexible polypeptide linker is not critical, however, it must be of a

sufficient length and flexibility such that the fragment domain and heterologous interactor domain fold independently and unhindered. The N-terminal, or α fragment library would be expressed as C-terminal fusions via a flexible polypeptide linker such as a $(Gly_4Ser)_3$ (SEQ ID NO:4) linker to the jun helix (Interactor 1 in Figure 6) from the *trc* promoter in the compatible pAE1 vector. Coding sequences for signal peptides would be included if translocation to the periplasm were desired.--

Please replace the paragraph beginning at page 29, line 6, with the following:

--This example demonstrates the ability of the system to detect and discriminate specific interactions between single-chain antibody Fv fragments (scFv) and 12-amino acid peptides ~~by~~ inserted into the active site of *E. coli* thioredoxin (trxpeps, Colas *et al.*, *Nature* (1996) *380*:548). ScFv are comprised of antibody heavy chain and light chain variable regions (VH and VL) tethered into a continuous polypeptide by most commonly a $(Gly_4Ser)_3$ (SEQ ID NO:4) linker encoded between most commonly the C-terminus of VH and the N-terminus of VL.--

Please replace the paragraph beginning at page 29, line 13, with the following:

--ScFv from a human non-immune antibody repertoire were amplified by PCR using a consensus primer mix (Marks *et al.*, *Eur J Immunol* (1991) *21*:985), and subcloned into a pUC119-based phagemid vector (Sambrook *et al.*, *supra*) for expression of the scFv as fusions to the N-terminus of the ω198 fragment with an intervening $(Gly_4Ser)_3$ (SEQ ID NO:4) linker (pAO1; see Figure 6A). An N-terminal signal peptide was provided for translocation to the bacterial periplasm. A commercial trxpep library was obtained and amplified by PCR using primers specific for the N- and C-termini of *E. coli* thioredoxin (Genbank accession no. M54881). This product was subcloned into a p15A replicon (Rose, *Nuc Acids Res* (1988) *16*:355) for expression as fusions to the C-terminus of the α197 fragment from the *trp-lac* fusion promoter (pAE1; see Figure 6B).

Again, an N-terminal signal peptide was provided for translocation to the periplasm. Figure 7 illustrates the activation of TEM-1 by complementation of α197 and ω198, mediated by interaction between an scFv and a trxpep.--

Please replace the paragraph beginning at page 31, line 11, with the following:

--This example demonstrates the ability of the system to work with larger antibody fragments, such as Fab, which are comprised of entire light chains disulfide-bonded to Fd fragments which contain VL plus the first heavy chain constant region. A subset of Fabs from a human repertoire library was subcloned for expression as C-terminal ω198 fusions from a dicistronic transcript from the *lac* promoter in the pAO1 vector (see Figure 6A). The first cistron encoded the light chain with a signal peptide for translocation to the periplasm. The light chain termination codon was followed by a short spacer sequence and then a ribosome binding site approximately 10 bp upstream from the start of translation for the signal peptide of the Fd fragment, which was followed by ω198 with an intervening $(Gly_4Ser)_3$ (SEQ ID NO:4) linker. This construct was then co-expressed with the α197-trxpep library in the pAE1 vector in strains DH5α and TG1. Spontaneous association of the light chain with the Fd-ω198 fusion protein in the periplasm was expected to produce a functional Fab fragment. Binding of the latter to the peptide on a α197-trxpep fusion was then expected to facilitate assembly of the functional TEM-1 β-lactamase in amounts sufficient to confer selectable resistance to ampicillin on the host cells.--

Please replace the paragraph beginning at page 33, line 7, with the following:

--This example demonstrates the ability of the present system to isolate panels of trxpeps that bind to a given protein of interest, and which could be used to map interaction surfaces on the protein, and which could also assist in the identification of new ligands by homology. The extra-cellular domain of the human B-cell activation

antigen CD40 is known to reliably express in the *E. coli* periplasm (Noelle *et al.*, *Immunol Today* (1992) *13*:431; Bajorath and Aruffo, *Proteins: Struct, Funct, Genet* (1997) *27*:59). A T-cell surface molecule, CD40 ligand (CD40L), is known to co-activate B-cells by ligation to CD40, but there may be other ligands. Therefore, TEM-1 α197/ω198 fragment complementation was used to select a panel of CD40-binding trxpeps. The sequences of these peptides would then be examined for homology to the known ligand and other potential ligands. The coding sequence for the mature form of the extra-cellular domain (CD40ED) was amplified by PCR using primers homologous to the N-terminus of the mature protein and to the C-terminus of the ~ 190-residue extra-cellular domain (Genbank accession no. X60592). The PCR product was then subcloned into the pAO1 phagemid vector (Figure 6A) for expression from the *lac* promoter as a C-terminal fusion to the TEM-1 ω198 fragment with an intervening $(Gly_4Ser)_3$ (SEQ ID NO:4) linker. Expression of the correct product was confirmed by PAGE, and the CD40 fusion vector was then rescued as phage and transfected into TG-1 cells bearing the same trxpep library construct as described above. Approximately 10^7 co-transformants were collected by double selection on kanamycin and chloramphenicol, and then plated onto 25μg/ml ampicillin. Activation of TEM-1 by a trxpep-CD40 interaction-mediated complementation of α197 and ω198 is depicted in Figure 8.--

Please replace the paragraph (**Table 2**) beginning at page 35, line 1, with the following:

--**Table 2**

Homologies of Representative CD40-binding Trxpeps

Group	TrxPep	Sequence[a]	SEQ ID NO:	Amp[r]
1	BW10-1	CGP**K**E**LRI**GGRPRRP**GP**C	8	+[b]
	P58-12-9A1	CGPEGQGG**V**A**V**GGVGGPC	9	+
	P65-2-4A2	CGPAK**RA**DVEFSL**E**PG	10	+
	CD40L	215-AKPCGQQSIHLGGVFELQPGA-235	11	
2	BW10-9	CGPKSAGKGRKD**RR**K**K**GPC	12	++
	P65-2-1A3	CG**P**R**T**RV**NH**QGQK**T**RGPC	13	+
	P65-2-2A5	**C**GPAGAIRHE**H**RQGLGPC	14	+
	CD40L	152-LVTLENGKQLTVKRQGLYYIYAQ-174	15	
3	P44-4-2A1	CGPDTGLETDA**AD**ASGPC	16	+
	P45-7-2A3	CGPRRVRETVA**VE**SSGPC	17	+
4	BW10-4	CGPPCA**T**FEEAKS**N**QGPC	18	+
	CD40L	104-ETKKENSFEMQKGDQNPQ-121	19	
5	P65-2-8A3	**C**GPG**R**ES**R**GRC**Y**TPSGPC	20	+
	CD40L	242-TDPSQVSHGTGFTSFGLL-259	21	
6	BW10-8	CGPNTPDEEMAPQAPGPC	22	++
7	P65-2-5A4	CGPVVHIKTNEQAAPGPC	23	+
8	P65-2-9A1	CGPVAEEPAGGAGRPGPC	24	+

[a]For sequence homologies, underlined denotes identity, bold denotes conservative substitution. For groups 1, 2, 4, and 5 homologies to CD40L only are depicted.

[b]Plating efficiencies when co-expressed with CD40-ω198 fusion on 25 μg/ml ampicillin. +, > 10% ; + + , > 50%.--

Please replace the paragraph beginning at page 43, line 13, with the following:

--The break-point disulfide overcomes a significant shortcoming of interaction-dependent enzyme fragment complementation systems. It is essential for high-throughput applications that such systems be capable of efficient activation by a wide range of heterologous protein-protein interactions. In other words, to minimize the false negative rate, the system must be activatable by any interaction between two proteins or fragments within the size range of single, naturally evolved protein domains, i.e., between ~ 100 and 300 amino acids in length. Globular proteins in this size range have radii in the range ~ 30-50Å. This means that the points of attachment for the linkers could be up to 100Å apart, and this distance must be spanned by the linkers in order for the break-points of the fragments to be able to come together. For this reason, the $(Gly_4Ser)_3$ (SEQ ID NO:4) linker was selected, which is expected to be fully extended and flexible, and to have a length of ~ 60Å, thereby providing a combined length of up to 120Å to allow close approach of the break-point termini during folding. Nevertheless, it is reasonable to expect the stability of the active conformation to be quite sensitive, and generally inversely proportional to the dimensions of the heterologous interaction. Thus, for all such systems described to date it may be assumed that the longer the linkers, the larger the proportion of possible interactions that can accommodate refolding, but the less the interaction can contribute to stabilization of the active conformation.--

Please replace the paragraph beginning at page 46, line 24, with the following:

--The GRE tri-peptide was also found to stabilize α197 in *trans*. When the α197-fos and jun-ω198 fusions were co-expressed in the *E. coli* periplasm with the GRE tri-peptide fused to the N-terminus of thioredoxin via a Gly_4Ser (SEQ ID NO:3) linker, the cells plated with 100% efficiency on 50 μg/ml ampicillin, whereas cells expressing the α197-fos and jun-ω198 fusions either alone, without the GRE-*trxA* fusion, or with a different tri-peptide-*trxA* fusion, plated with only ~ 1% efficiency on 50 μg/ml ampicillin.

The GRE-*trxA* fusion conferred no resistance to ampicillin in the absence of the interacting helixes, thus it does not stabilize the re-folded fragment complex, but rather it must stabilize the α197 fragment since activity is limited by the amount of soluble α197. Since the GRE tri-peptide had the same stabilizing effect on α197 fragment when a different carrier was used, its activity must be context independent. Thus, an 18 kDa enzyme fragment could be stabilized at least 100-fold by a tri-peptide selected from a random sequence library. As with the tethered tri-peptide, the free GRE tri-peptide could inhibit aggregation of α197 without apparently interfering with re-folding of the fragment complex. In this case, however, displacement of the tri-peptide would have been greatly assisted by the fact that the effective intra-molecular concentrations of structural elements relative to one another would have been much higher than the tri-peptide concentration. In this way the general ability of small peptides to stabilize large proteins in *trans* without interfering with protein folding may be understood. This phenomenon is not widely appreciated, and in fact this may be the first demonstration that a functional protein could be deliberately stabilized by something as small as a tri-peptide.--

Please replace the paragraph beginning at page 55, line 13, with the following:

--The first step in developing the Her-2/neu inactivation biosensor would be to obtain a Tyr1068-binding protein. This could be accomplished by inserting the coding sequence for the substrate peptide, PVPEYINQS (SEQ ID NO:25), into the active site of thioredoxin, between G33 and P34, flanked by short flexible linkers such as PGSGG (SEQ ID NO:26) to minimize structural constraints on the peptide, which does not require a rigid structure for binding to its natural ligand, the Grb2 SH2 domain. This Tyr1068 trxpep can then be fused via a $(Gly_4Ser)_3$ (SEQ ID NO:4) linker to the N-terminus of ω254, and co-expressed in *E. coli* TG-1 cells with a scFv library of at least 10^8 clones, or a VL library of at least 10^6 clones fused to the C-terminus of α253 via the $(Gly_4Ser)_3$ (SEQ ID NO:4) linker. Since the Tyr1068-binder is being selected for deployment in the mammalian cell cytoplasm, it might be prudent to perform the

selections in the *E. coli* cytoplasm. For this purpose the vectors in Figure 6 could be used with the signal peptides removed. Then a chromogenic substrate such as nitrocefin (λmax = 485 nm; ϵ = 17,420 M^{-1} cm^{-1}; McManus-Munoz and Crowder, *Biochemistry* (1999) *38*:1547) would be used to select Tyr1068-binders by color. By plating at least 10^6-10^8 transformants at moderate to high stringency, i.e., on decreasing concentrations of the substrate, it should be possible to identify binders with sub-micromolar affinities since Tyr is the most common amino acid in high-affinity protein-protein interfaces. Such affinities will be desirable for maximum discrimination between Tyr and phospho-Tyr. Selected Tyr1068-binders must be tested for inhibition by phosphorylation of the Tyr. This can easily be accomplished by expressing the vectors in isogenic cells which over-express a broad spectrum Tyrosine kinase (TKX1 cells, Stratagene, Inc., La Jolla, CA).--

Please replace the paragraph beginning at page 56, line 3, with the following:

--Once a suitable phosphate-sensitive Tyr1068-binder has been identified, the entire coding sequence for the α253 – Tyr1068-binder fusion may be subcloned into a mammalian expression vector, such as the pCMV-Tag vectors (TKX1 cells, Stratagene, Inc., La Jolla, CA) for expression in mammalian cells from the cytomegalovirus promoter. The ω254 fragment must be expressed as a fusion to the C-terminus of the Her-2/neu cytoplasmic domain, which contains Tyr1068. The coding sequence of the 1210-residue EGF receptor (Genbank accession no. X00588; Ullrich *et al.*, *Nature* (1984) *309*:418) may be used as it is operationally identical to Her-2/neu, and its Tyr1068 will become phosphorylated under the same conditions of over-expression and/or growth factor ligation in tumor cells. When fused to the C-terminus of EGFR via the $(Gly_4Ser)_3$ (SEQ ID NO:4) linker, the 35-residue ω254 β-lactamase fragments will be only 152 residues away from Tyr1068. Both the EGFR-ω254 fusion and the α253-Tyr1068-binder fusion may be expressed from the same vector from a dicistronic mRNA. This is accomplished by inserting an internal ribosome entry site (IRES; Martinez-Salas; *Curr*

Opin Biotechnol (1999) *10*:458) between the termination codon of the upstream cistron and the initiation codon of the downstream cistron. This will allow both proteins to be made simultaneously from the same mRNA. The vector may be introduced into the tumor cell line by cationic liposome-mediated transfection, using e.g., lipofectamine (Gibco-BRL, Gaithersburg, MD) according to the protocol in the product literature. Operation of the biosensor may be tested in transiently transfected cells, and if operational, stable transformants may then be isolated by selection for long term antibiotic resistance. Multiple free-diffusible chromogenic and fluorogenic substrates are available for continuous monitoring of β-lactamase activity. Operationally, the ω254 fragment will be anchored to the plasma membrane at the C-terminus of the cytoplasmic domain of the receptor near Tyr1068, and the α253 fragment will be free in the cytoplasm as the Tyr1068-binder fusion. ATP-analog tyrosine kinase inhibitors are available commercially and can be used as positive controls for inhibitor selection, and to determine the signal increment from fully-activated to fully-inhibited EGFR.--

Please replace the paragraph beginning at page 57, line 14, with the following:

--For a two fragment system, dependence of activation on the interaction of heterologous domains is not necessary. However, for simultaneous selection of triple transgenics, complementation of the enzyme fragment pair must be dependent on a heterologous interaction mediated by a free ligand, analogous to the activation of β-lactamase by the tri-molecular interaction of α197-jun, scFv-ω198, and CD40-fos, as described above. For these applications, the most important parameter is the maximum activity of the reconstituted enzyme, which is a function of both the specific activity and the efficiency of complementation. The activation index is not relevant because the each fragment alone will have essentially no detectable activity, providing a background of zero. Thus, to insure recovery of the most competent fragment pairs for intra-cellular activity, the fos and jun interactors should be used with tri-peptide libraries between the break-points and the $(Gly_4Ser)_3$ (SEQ ID NO:4) linkers. The tri-peptide libraries will

provide stabilizers for each fragment so that the selection will be biased toward the fragments producing the highest specific activities. For two-trait selection applications, i.e., bi-molecular selections, where a heterologous interaction is not required, specific activity may be increased further by mutagenesis and selection for fold accelerating mutations. For three-trait selection applications, selected fragment pairs will have to be tested for dependence on the heterologous interaction. In this case, the activation index will be of some importance, but as with in vitro applications a modest index of 1000 will be more than adequate for clean selections.--

Please replace the paragraph beginning at page 61, line 28, with the following:

--The tumor activation mechanism for these fragments may employ two scFvs such as those described by Schier *et al.* (*Gene* (1996) *169*:147), which were derived from a phage display library of a human non-immune repertoire (Marks et al., 1991) by panning against a recombinant fragment comprising the extra-cellular domain (ED) of Her-2/neu. These two scFv; appear to recognize non-overlapping epitopes, since they do not compete for binding to the Her-2/neu_ED by ELISA. The affinity of one of these scFv was improved to sub-nM Kd in vitro (Schier *et al.*, 1996, *supra*), and similar improvements in the other could be made using the same methods (Balint and Larrick, *Gene* (1993) *137*:109). The coding sequences for the scFv may be subcloned into the β-lactamase α and ω fusion production vectors, pβlacα and pβlacω, shown in Figure 11. These vectors are derived from pET26b (Novagen), and have convenient restriction sites for insertion of both scFv and β-lactamase fragment sequences. Each fusion protein is inducibly expressed (IPTG) from the strong phage T7 promoter under the control of the *lac* repressor. Each primary translation product contains a pelB signal peptide for secretion into the bacterial periplasm and a C-terminal His_6 (SEQ ID NO:5) tag for one-step purification from osmotic shock extracts by immobilized metal ion affinity chromatography (IMAC, Janknecht *et al.*, *Proc Natl Acad Sci* (1991) *88*:8972). The

yield of each fusion protein can be optimized primarily by manipulation of the inducer concentration and the growth temperature.--

Please replace the paragraph beginning at page 62, line 14, with the following:

--Each scFv may be expressed as both α and ω fusions to determine which arrangement(s) (1) support the highest binding activity, (2) support the highest enzymatic activity, and (3) support the highest yields. Initially, expression may be optimized by the criterion of silver-stained PAGE. Then fusion proteins should be purified from osmotic shock extracts (Neu and Heppel, 1965, *supra*) by IMAC. The purified fusion proteins may be tested for binding to an immobilized recombinant fusion of the Her-2/neu extracellular domain (ED) to a stabilizing immunoglobulin domain (Ig) by ELISA using an anti-His_6 (SEQ ID NO:5) tag antibody (Qiagen). The purified fusion proteins may then be tested for reconstitution of β-lactamase activity on immobilized rc- Her-2/neu ED-Ig using a chromogenic substrate, nitrocefin (λmax = 485 nm; ϵ = 17, 420 M^{-1} cm^{-1}; McManus-Munoz and Crowder, 1999, *supra*). Immobilized BSA may be used as the negative control.--

Please insert the accompanying paper copy of the Sequence Listing, page numbers 1 to 10, at the end of the application.

REMARKS

Applicants request entry of this amendment in adherence with 37 C.F.R. §§1.821 to 1.825. This amendment is accompanied by a floppy disk containing the above named sequences, SEQ ID NOS:1-26, in computer readable form, and a paper copy of the sequence information which has been printed from the floppy disk.